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Loss of Sprouty2 in human high-grade serous ovarian carcinomas promotes EGF-induced E-cadherin down-regulation and cell invasion ☆

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ABSTRACT

Sprouty (SPRY) proteins are well-characterized factors that inhibit receptor tyrosine kinase signaling. Our Human Exonic Evidence-Based Oligonucleotide (HEEBO) microarray results showed that the mRNA levels of SPRY2, but not of SPRY1 or SPRY4, are down-regulated in high-grade serous ovarian carcinoma (HGSC) tissues and epithelial ovarian cancer (EOC) cell lines. Molecular inversion probe (MIP) copy number analysis showed the deletion of the *SPRY2* locus in HGSC. Overexpression of SPRY2 reduced EGF-induced cell invasion by attenuating EGF-induced E-cadherin down-regulation. Moreover, a positive correlation between SPRY2 and E-cadherin protein levels was observed in HGSC tissues. This study reveals the loss of SPRY2 in HGSC and indicates an important tumor-suppressive role for SPRY2 in mediating the stimulatory effect of EGF on human EOC progression.

1. Introduction

Ovarian cancer is the fifth leading cause of all female cancer-related deaths and the most lethal gynecologic malignancy in North America. Approximately 60% of women who develop ovarian cancer will die from the disease [1,2]. Epithelial ovarian cancers (EOCs) are divided into several histologic subtypes based on tumor morphology [3]. The four major subtypes are serous, endometrioid, clear cell and mucinous. Among these major subtypes, high-grade serous carcinoma (HGSC) is the most common [4]. Although EOC includes a majority of all ovarian carcinomas, its origin and etiology have not yet been completely elucidated. It has been shown that a malfunction of receptor tyrosine kinases (RTKs), including epidermal growth factor receptor (EGFR), contributes to the development of EOC [5]. EGFR and its ligands play a critical role in the stimulation of cancer cell proliferation, survival and metastasis [6,7], and the overexpression of EGFR ligands has been detected in human ovarian cancer tissue and ascitic fluid [8,9]. Moreover, EGFR levels were found to be up-regulated in the advanced stage of the disease and in metastases [6].

In addition to RTK abnormalities, the loss of endogenous regulators represents an alternative mechanism that leads to aberrant RTK activity. Sprouty (SPRY), which acts as an antagonistic regulator of tracheal branching, was first identified in *Drosophila* [10]. To date, four *SPRY* genes (*SPRY1–4*) have been identified in mammals [11]: *SPRY1*, *SPRY2* and *SPRY4* are expressed in various mouse embryonic tissues, whereas *SPRY3* is only detected in the adult brain and testis [11,12]. Similar to *Drosophila* SPRY, mammalian SPRY proteins are able to negatively regulate various growth factors that induce RTK signaling by inhibiting the RAS/ERK1/2 pathway [13,14]. Different *SPRY*s have been found to be down-regulated in several human cancers, including breast, prostate, liver and lung cancers [15]. Functionally, SPRY has been reported to interfere with the cell cycle, cell proliferation, migration and invasion, in vitro and in vivo tumorigenic potential, and the formation of metastases [16–21]. Importantly, the prognostic value of the level of SPRY has been established in renal, liver and prostate

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cancer patients [22–24]. Moreover, expression of SPRY4 serves as a reliable marker of the response to Gleevec treatment for patients with gastrointestinal stromal tumors [25]. Nonetheless, the mechanism by which SPRY is down-regulated appears to vary depending on the cancer type, and contradicting data exist for similar malignancies [16–19,23,24,26,27].

The present study aimed to investigate the underlying mechanisms and functions of SPRY in human EOC, in addition to SPRY down-regulation. We demonstrate that the mRNA levels of SPRY2, but not SPRY1 or SPRY4, are significantly down-regulated in both human HGSC tissues and EOC cell lines. Moreover, the *SPRY2* gene is deleted in some HGSC tissues, which may, in part, cause the down-regulation of SPRY2 levels. We further show that the overexpression of SPRY2 in a human ovarian cancer cell line, SKOV3, attenuates the EGF-induced down-regulation of E-cadherin and cell invasion. Furthermore, the protein levels of SPRY2 are positively correlated with the protein levels of E-cadherin in HGSC tissues.

2. Materials and methods

2.1. The Human Exonic Evidence-Based Oligonucleotide (HEEBO) microarray

The HEEBO microarray (Stanford, CA, USA) employed for this study includes 44544 70-mer probes that were designed using transcriptome-based annotation of the exonic structure of genomic loci. Pooled RNA from 10 human cancer cell lines of different origins (Stratagene, Universal Human Reference RNA, Catalog number: 740000) for broad gene coverage on the array was included as a reference. We examined the mRNA expression profiles of a series of ovarian tumors from the Vancouver General Hospital (Vancouver, BC, Canada) tumor bank obtained from patients who underwent surgery in 2004 and 2005. These cases included the following: high-grade serous ($n = 35$), low-grade serous ($n = 2$), endometrioid ($n = 7$), clear cell ($n = 3$), serous borderline ($n = 1$), endometrioid borderline tumor ($n = 1$) and normal fallopian tube ($n = 1$) subtypes. Approval for the study was obtained from the University of British Columbia Research Ethics Board (#H04-60102), and written informed consent was obtained from all participants involved in the study. The HEEBO microarray analysis was performed as described previously [28].

2.2. Molecular inversion probe (MIP) copy number analysis

To determine whether deletion of the *SPRY2* and *SPRY4* genes occurs in human ovarian tumors, an MIP copy number analysis was performed using samples from another cohort of patients. All women who underwent primary debulking surgery at the Vancouver General Hospital and British Columbia Cancer Agency in Vancouver, Canada, between January 2004 and September 2005 were invited to participate in the analysis, except those with mucinous and borderline tumors or who had received pre-operative chemotherapy. The pathology data were reviewed by a pathologist (Dr. C. Blake Gilks). The classification and grading of tumors were performed as described previously [29]. We included 28 high-grade serous, 5 high-grade serous/undifferentiated, 3 high-grade undifferentiated, 5 endometrioid, 4 clear cell and 1 low-grade serous carcinomas. Ethical approval was obtained from the University of British Columbia Research Ethics Board (#H02-61375 and #H03-70606), and written informed consent was obtained from all participants involved in the study. The MIP copy number assay and copy number estimation were performed as described previously [30]. A copy number over 3.0 was considered an amplification event, and a copy number below 1.5 was considered a deletion event.

2.3. The cancer genome atlas (TCGA)

To obtain direct evidence that the reduced SPRY2 mRNA levels were dependent on gene deletion, we retrieved data from the TCGA database portal (http://cancergenome.nih.gov/). Copy number data for 585 ovarian serous cystadenocarcinomas and 587 normal samples (569 matched and 18 unmatched) were extracted. For the gene expression profile, data from 584 tumors and 18 unmatched normal samples were extracted.

2.4. Cell culture and reagents

Four non-tumorigenic SV40 Tag immortalized ovarian surface epithelial cell lines (IOSEs), including IOSE-29, IOSE-80, IOSE-120, and IOSE-398, were generous gifts from Dr. Nelly Auersperg (University of British Columbia) [31]. The human ovarian adenocarcinoma cell line BG-1 was kindly provided by Dr. K.S. Korach (National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC, USA) [32]. The CaOV3, OVCAR3 and SKOV3 ovarian cancer cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in MCDB 105/M199 (1:1) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 g/ml streptomycin. The cells were cultured at 37 °C with 5% CO_2. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT, USA). Human recombinant EGF and other tissue culture materials were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. To overexpress human SPRY2, the pXJ40-FLAG-SPRY2 vector and empty pXJ40-FLAG vector (gifts from Dr. Graeme R. Guy, Institute of Molecular and Cell Biology, Singapore) [33] were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.5. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using an ABI 7300 real-time thermal cycler (ABI, Hercules, CA, USA). SPRY1, SPRY2, SPRY4 and the internal control GAPDH were amplified in duplicate with the following PCR primers: SPRY1, forward 5′-ATG GAT CCC CAA AAT CAA CA-3′ and reverse 5′-CGA GGA GCA GGT CTT TTC AC-3′; SPRY2, forward 5′-CCC CTC TGT CCA GAT CCA TA-3′ and reverse 5′-CCC AAA TCT TCC TTG CTC AG-3′; SPRY4, forward 5′-AGC CTG TAT TGA GCG GTT TG-3′ and reverse 5′-GGT CAA TGG GTA GGA TGG TG-3′, E-cadherin, forward 5′-ACA GCC CCG CCT TAT GAT T-3′ and reverse 5′-TCG GAA CCG CTT CCT TCA-3′; SNAIL, forward 5′-CCC CAA TCG GAA GCC TAA CT-3′ and reverse 5′-GCT GGA AGG TAA ACT CTG GAT TAG A-3′; SLUG, forward 5′-TTC GGA CCC ACA CAT TAC CT-3′ and reverse 5′-GCA GTG AGG GCA AGA AAA AG-3′; and GAPDH, forward 5′- GAG TCA ACG GAT TTG GTC GT-3′ and reverse 5′-GAC AAG CTT CCC GTT CTC AG-3′.

2.6. Western blotting analysis

Equal amounts of total cell lysate were resolved on 10% SDS–PAGE gels and electrotransferred onto a PVDF membrane. After blocking for 1 h with 5% non-fat dry milk in TBS-T buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), the blots were probed overnight at 4 °C with primary antibodies. The anti-E-cadherin antibody was obtained from BD Transduction Laboratories (Lexington, KY, USA). The anti-SPRY2 antibody was purchased from Sigma. The anti-SNAIL and anti-SLUG antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). The anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The blots were then incubated with a peroxidase-conjugated secondary antibody (Bio-Rad) for 1 h, followed by detection with an ECL chemiluminescence reagent and exposure to X-ray films.

2.7. Invasion assay

Twenty-four-well Transwell filters with an 8-μm pore size coated with 1 mg/ml Matrigel (50 μl/well; BD Sciences, Mississauga, ON, Canada) were used to assess cell invasion. SKOV3 cells transfected with either control or SPRY2-overexpression constructs were trypsinized and resuspended in 0.1% FBS medium, with or without 100 ng/ml EGF, and then seeded in triplicate in the upper chamber. A medium containing 1% FBS was added to the lower wells. The chambers were incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2. The cells that did not penetrate the filter were wiped off. The invading cells on the lower surface of the filter were fixed with ice-cold methanol, stained with Hoechst 33258 and counted via epifluorescence microscopy with Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON, Canada). Triplicate inserts were used for each individual experiment, and the results are presented as the mean numbers.

2.8. Statistical analysis

A statistical analysis was performed using Prism graphing software. Differential variation in SPRY mRNA levels among ovarian tumor subtypes was assessed using the Kruskal–Wallis rank test followed by Student's *t* test to compare each tumor subtype. The relative quantification of mRNA expression levels, as assessed by RT-qPCR, was calculated using the $2^{-\Delta\Delta}$ Ct method. For the invasion assay and the comparison of genes or proteins expression levels with the control (using SPRY2-overexpressing cells), a one-way ANOVA and non-parametric column analysis was performed followed by Tukey's multiple comparison test to compare all pairs of columns. Columns with different letters are significantly different ($P < 0.05$). The data are presented as the mean ± SD of three or four independent experiments. The Pearson correlation coefficient (r) and associated probability (P) were calculated when comparing E-cadherin and SPRY2 protein levels using the Spearman non-parametric correlation.

3. Results

3.1. Levels of SPRY mRNA in different pathological subtypes of human EOC

To examine whether the mRNA levels of SPRY isoforms are down-regulated in human EOC, we compared EOC samples of various histopathological subtypes, including serous (high-grade, low-grade or borderline), endometrioid (carcinoma or borderline tumor) and clear cell subtypes. The results of the HEEBO microarray analysis showed no significant differences for the mRNA levels of three different SPRY1 transcripts in each subtype (Kruskal–Wallis test, $P = 0.396$, 0.706 and 0.813). Similarly, SPRY4 mRNA levels were not significantly different between the subtypes ($P = 0.33$). Interestingly, significant differences in the SPRY2 mRNA levels were observed in various tumors types ($P = 0.0091$). The lowest mean level of SPRY2 mRNA was observed in the clear cell subtype, followed by HGSC; all other samples displayed higher mean SPRY2 mRNA levels compared with the reference level (Fig. 1). When comparing pathological subtypes, the difference in SPRY2 mRNA levels between high-grade and low-grade serous subtypes was statistically significant ($P = 0.022$). The mean SPRY2 mRNA levels in serous and clear cell carcinomas were statistically lower than that of endometrioid carcinomas (serous vs. endometrioid, $P = 0.0007$; clear cell vs. endometrioid, $P = 0.022$).

3.2. Levels of SPRY mRNA in cultured human EOC cell lines

Next, we examined SPRY mRNA levels in four human EOC cell lines (BG-1, CaOV3, OVCAR3 and SKOV3) by RT-qPCR. Four immortalized human ovarian surface epithelial cell lines (IOSEs) established from individual patients were included for comparison. As shown in Fig. 2, our results showed that the mRNA level of SPRY1 was higher in all human EOC cell lines compared with the IOSE cell lines and that the SPRY4 level was consistently lower in the EOC cell lines. Down-regulated SPRY2 mRNA levels were detected in three out of four human EOC cell lines.

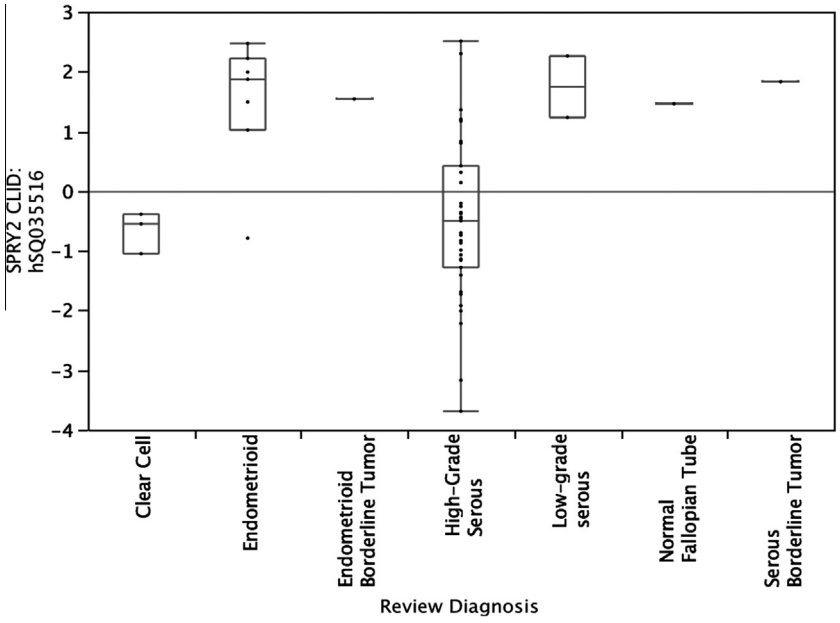

Fig. 1. The box plot displays the HEEBO microarray results for SPRY2 mRNA levels in human EOC of various subtypes: high-grade serous ($n = 35$), low-grade serous ($n = 2$), endometrioid ($n = 7$), clear cell ($n = 3$), serous borderline ($n = 1$), endometrioid borderline tumors ($n = 1$) and normal fallopian tube ($n = 1$). Pooled RNA from 10 human cancer cell lines of different origins for broad gene coverage on the array was included as a reference (0). Dots represent individual samples.

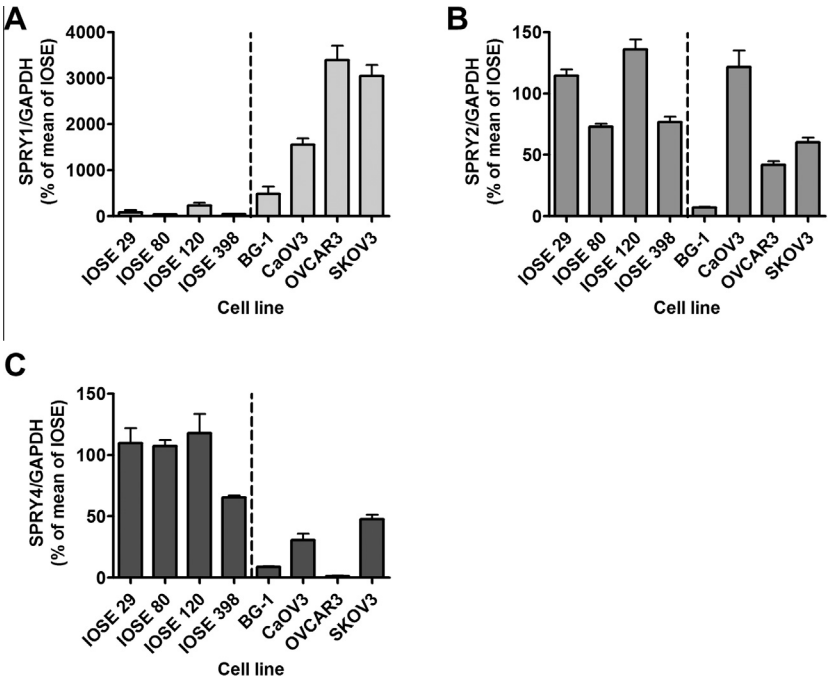

Fig. 2. Comparison of SPRY1, SPRY2 and SPRY4 mRNA levels, as measured by RT-qPCR, in human immortalized OSE (IOSE) and EOC cell lines. The SPRY1, SPRY2 and SPRY4 mRNA levels were normalized against the internal control GAPDH. The average normalized expression levels of SPRY1, SPRY2 and SPRY4 in the IOSE cell lines were calculated and set to 100%. The relative levels of SPRY1 (A), SPRY2 (B) and SPRY4 (C) mRNA levels in EOC cell lines are expressed as a percentage of their mean expression in IOSE cell lines. The results are expressed as the means ± SD of three independent experiments from three passages.

3.3. Deletion of the SPRY2 and SPRY4 loci in human EOC

An MIP copy number analysis was performed to examine to the occurrence of chromosomal changes in the human *SPRY2* and *SPRY4* genes. Because there are no specific intragenic probes available, we used probes closely flanking the *SPRY2* and *SPRY4* loci as surrogate markers. Among the 46 EOC tissues analyzed, two markers tightly flanking the *SPRY2* locus (MIP ID: 437874 and MIP ID: 440961) revealed loss in 23.4% (11/46) and 19.6% (9/46), respectively, of the samples. Notably, such deletion events were most frequent in HGSC, with frequencies of 32.1% (9/28) to 35.7% (10/28) (Fig. 3). Conversely, none of the endometrioid, clear cell, or low-grade serous tumors exhibited *SPRY2* gene deletion (Table 1). Two additional markers (MIP ID: 437922 and MIP ID: 442871) revealed similar frequencies of loss (25% and 35.7%, respectively) in HGSC (Fig. 3). In contrast, the deletion of the markers closest to the *SPRY4* locus (MIP ID: 431289 and MIP ID: 439693) was rarely found in HGSC, with deletion only found in 3.6% (1/28) and 7.1% (2/28), respectively, of the samples (Fig. 3). Two markers more distant from the *SPRY4* gene (MIP ID: 431174 and MIP ID: 448347) revealed only 7.1% (2/28) and 3.6% (1/28) loss, respectively (Fig. 3). In a TCGA data set, 24% of the ovarian serous cystadenocarcinoma samples displayed a decrease in gene copy number, and a majority (67%) of these samples showed a decrease in the SPRY2 mRNA level. In contrast, 54% of the ovarian tumors in the TCGA gene expression analysis showed a decrease in the SPRY2 level, though only 32% of these tumors also showed a decrease in copy number. Taken together, these results indicated that *SPRY2* gene deletion might, in part, contribute to reduced SPRY2 expression and also suggested that additional mechanisms might contribute to reduced SPRY2 expression in HGSC.

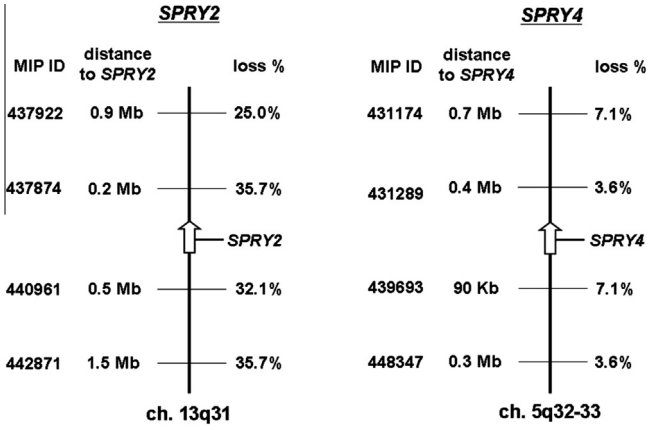

Fig. 3. A schematic representation of the MIP copy number assay results for 28 high-grade serous ovarian carcinomas. The diagram shows the MIP ID, distance to the *SPRY* locus and corresponding percentage of the loss of markers flanking the *SPRY2* locus (left panel) and markers flanking the *SPRY4* locus (right panel).

3.4. Overexpression of SPRY2 attenuates EGF-induced down-regulation of E-cadherin and up-regulation of SNAIL in human ovarian cancer cells

Our previous studies have shown EGF-induced cell invasion via the ERK1/2-mediated down-regulation of E-cadherin in the human ovarian cancer cell line SKOV3 [34,35]. Given the important role of SPRY2 in the regulation of ERK1/2 signaling, we examined the function of SPRY2 in EGF-induced down-regulation of E-cadherin and cell invasion. As shown in Fig. 4A, consistent with our previous results, treatment of SKOV3 cells with 100 ng/ml EGF for 24 h significantly down-regulated E-cadherin mRNA levels. Interestingly, SPRY2 overexpression did not affect basal E-cadherin mRNA levels but did attenuate the down-regulation induced by EGF treatment (Fig. 4A). Similarly, Western blotting results showed that treatment with 100 ng/ml EGF for 24 h down-regulated E-cadherin protein levels and that SPRY2 overexpression attenuated the EGF-induced down-regulation of E-cadherin protein levels (Fig. 4B). It has been well characterized that the down-regulation

Table 1
MIP analysis of loss of markers flanking *SPRY2* and *SPRY4* loci in ovarian tumors.

Number	Histopathology	SPRY2				SPRY4	
		Proximal 2	Proximal 1	Distal 1	Distal 2	Proximal	Distal
223	Serous HG	**1.40**	**1.40**	1.58	1.58	2.09	1.92
329	Serous HG	**1.37**	**1.20**	**1.26**	**1.26**	**1.44**	**1.33**
293	Serous HG	1.94	1.88	1.88	1.88	2.10	2.22
283	Serous HG	**1.44**	**1.09**	**1.30**	**1.30**	2.43	2.13
239	Serous HG	2.41	2.24	2.28	2.28	2.42	2.31
327	Serous HG	2.26	1.98	2.49	2.13	2.31	2.07
379	Serous HG	1.58	1.58	**1.41**	**1.36**	2.09	2.09
163	Serous HG	**1.46**	**1.43**	**1.44**	**1.44**	1.77	1.99
305	Serous HG	2.09	2.09	1.94	1.94	2.61	2.32
212	Serous HG	1.54	2.76	1.54	2.12	1.83	2.36
330	Serous HG	2.34	2.17	2.57	2.56	1.56	**1.41**
332	Serous HG	2.08	1.69	**1.30**	**1.30**	2.12	1.77
388	Serous HG	2.97	2.97	3.01	2.56	2.05	1.94
363	Serous HG	1.57	**1.36**	**1.36**	**1.32**	2.21	2.27
344	Serous HG	2.06	1.57	2.26	1.74	2.73	2.44
345	Serous HG	**1.29**	**1.28**	**1.18**	**1.18**	2.47	2.91
384	Serous HG	1.93	1.82	1.97	1.74	2.71	2.36
178	Serous HG	2.04	2.02	2.02	1.57	2.67	2.35
229	Serous HG	**1.35**	**1.35**	1.53	**1.46**	2.26	2.10
309	Serous HG	2.36	2.36	2.45	2.21	2.23	2.23
394	Serous HG	1.96	1.83	1.83	1.82	2.22	2.09
195	Serous HG	1.75	**1.24**	**1.20**	**1.20**	2.39	2.39
236	Serous HG	**1.42**	**1.42**	1.53	1.53	1.59	1.90
172	Serous HG	2.75	2.22	2.13	1.99	1.62	1.55
254	Serous HG	1.68	1.68	1.75	1.84	2.05	2.05
319	Serous HG	2.51	2.16	2.30	2.30	2.15	2.21
372	Serous HG	1.50	**1.36**	**1.36**	**1.30**	2.35	2.41
297	Serous HG	2.03	2.00	2.09	1.86	2.27	2.19
208	Undifferentiated HG	3.03	2.53	2.72	2.72	1.81	1.81
273	Undifferentiated HG	1.67	**1.48**	1.54	1.54	1.94	1.66
240	Undifferentiated HG	2.27	2.10	2.27	2.30	2.10	2.02
161	Serous/undifferentiated HG	1.87	1.72	1.67	1.67	1.87	1.82
336	Serous/undifferentiated HG	1.88	2.27	1.83	1.83	1.87	1.87
186	Serous/undifferentiated HG	2.44	2.09	2.09	2.36	2.60	2.72
201	Serous/undifferentiated HG	2.23	2.23	1.81	1.81	3.24	3.24
280	Serous/undifferentiated HG	2.62	2.57	2.54	2.54	1.89	2.25
198	Clear cell	2.18	2.01	2.23	1.88	3.04	2.42
213	Clear cell	2.14	2.14	1.86	1.86	2.01	1.98
219	Clear cell	2.48	2.50	2.61	2.61	2.26	2.07
392	Clear cell	2.04	1.81	2.04	1.64	2.54	2.31
242	Endometrioid – G2	2.37	2.02	2.37	1.91	2.58	2.14
281	Endometrioid – G2	2.23	2.41	2.45	2.45	2.23	2.23
334	Endometrioid – G1	2.02	1.94	2.32	2.23	2.35	1.92
156	Endometrioid – G2	1.93	1.93	1.90	1.90	2.18	1.88
343	Endometrioid – G2	2.46	2.03	2.03	2.03	2.26	2.33
324	Serous LG	2.00	1.97	2.00	2.00	1.92	2.13

MIP analysis were performed on 28 high-grade serous, 5 high-grade serous/undifferentiated, 3 high-grade undifferentiated, 5 endometrioid, 4 clear cell and 1 low-grade serous ovarian tumors to detect presence of the marker flanking the *SPRY2* and *SPRY4* loci. The MIP copy numbers below 1.5 were considered deletion events (bolded). HG: high-grade; LG: low-grade; G1: grade 1; G2: grade 2; G3: grade 3.

of E-cadherin expression can be achieved by SNAIL- and SLUG-mediated transcriptional suppression [36]. As shown in Fig. 4C, treatment with 100 ng/ml EGF for 3 h significantly up-regulated SNAIL and SLUG mRNA levels, whereas the overexpression of SPRY2 did not affect the basal mRNA levels of SNAIL and SLUG. Interestingly, SPRY2 overexpression only attenuated the EGF-induced up-regulation of SNAIL mRNA levels but not those of SLUG (Fig. 4C). Similarly, treatment with 100 ng/ml EGF for 3 h significantly up-regulated SNAIL and SLUG protein levels, and the overexpression of SPRY2 attenuated the EGF-induced up-regulation of SNAIL protein levels but not those of SLUG (Fig. 4D).

3.5. Overexpression of SPRY2 attenuates EGF-induced human ovarian cancer cell invasion

We have shown that EGF treatment induces human ovarian cancer cell invasion by down-regulating E-cadherin expression and that E-cadherin overexpression blocks EGF-induced human ovarian cancer cell invasion [34,35,37,38]. Because the overexpression of SPRY2 attenuated the EGF-induced down-regulation of E-cadherin, we next examined whether it could also affect EGF-induced ovarian cancer cell invasion. As shown in Fig. 5, SPRY2 overexpression attenuated EGF-induced cell invasion without affecting basal cell invasiveness.

3.6. SPRY2 expression levels are positively correlated with the expression levels of E-cadherin in human EOC

To further examine the correlation between SPRY2 and E-cadherin, we analyzed the protein levels of SPRY2 and E-cadherin in eleven HGSC tissues isolated from patients. As shown in Fig. 6A, the SPRY2 and E-cadherin proteins were detected in the majority of samples by the results of Western blotting. In addition, the results of a Spearman non-parametric correlation analysis revealed a statistically significant positive correlation (Pearson correlation coefficient, $r = 0.6620$ and $P = 0.0265$) (Fig. 6B).

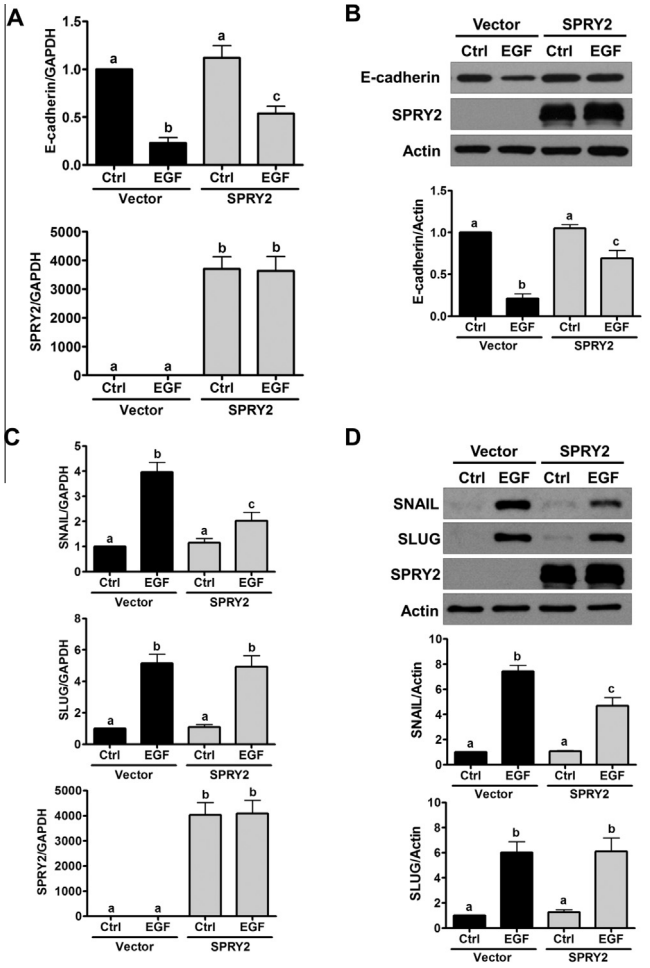

Fig. 4. The effect of SPRY2 overexpression on EGF-induced up-regulation of SNAIL and SLUG and down-regulation of E-cadherin. (A) and (B) SKOV3 cells were transfected with the empty pXJ40-FLAG vector (Vector) or SPRY2-overexpression pXJ40-FLAG-SPRY2 vector (SPRY2) for 48 h and then treated with vehicle control (Ctrl) or 100 ng/ml EGF for 24 h. The mRNA (A) and protein (B) levels of E-cadherin and SPRY2 were analyzed by RT-qPCR and Western blotting, respectively. (C) and (D) SKOV3 cells were transfected with the empty pXJ40-FLAG vector (Vector) or SPRY2-overexpression pXJ40-FLAG-SPRY2 vector (SPRY2) for 48 h and then treated with vehicle control (Ctrl) or 100 ng/ml EGF for 3 h. The mRNA (C) and protein (D) levels of SNAIL, SLUG and SPRY2 were analyzed by RT-qPCR and Western blotting, respectively. The results are expressed as the mean ± SEM from at least three independent experiments. Values without a common letter are significantly different ($P < 0.05$).

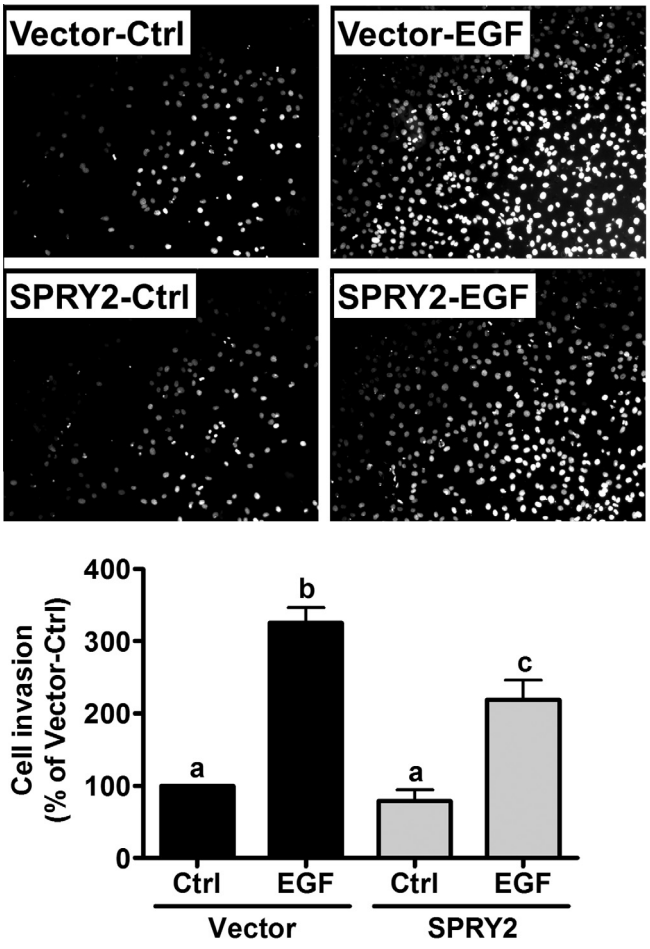

Fig. 5. The effect of SPRY2 overexpression on EGF-induced human ovarian cancer cell invasion. SKOV3 cells were transfected with the empty pXJ40-FLAG vector (Vector) or SPRY2-overexpression vector pXJ40-FLAG-SPRY2 (SPRY2) for 48 h. The cells were treated with vehicle control (Ctrl) or 100 ng/ml EGF and then seeded into Matrigel-coated Transwell inserts and cultured for an additional 24 h. Non-invading cells were removed from the upper side of the filter, and the nuclei of the invaded cells were stained with Hoechst 33258. The top panel shows representative fluorescence images from the invasion assay. The bottom panel summarizes the quantitative results, which are expressed as the mean ± SEM from three independent experiments. Values with different letters are significantly different ($P < 0.05$).

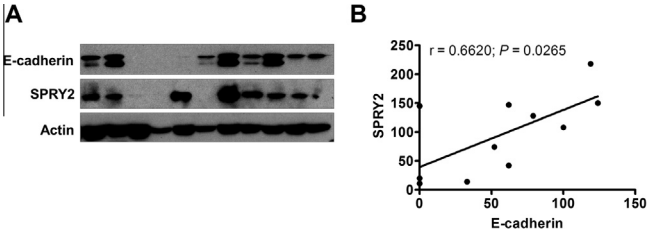

Fig. 6. The correlation between SPRY2 and E-cadherin protein levels in human EOC tissues. (A) The protein levels of E-cadherin and SPRY2 in eleven high-grade serous ovarian carcinomas were analyzed by Western blotting. (B) The protein expression levels of E-cadherin and SPRY2 were quantified, and correlations were assessed using the Spearman non-parametric correlation method.

4. Discussion

SPRY proteins have been identified as endogenous inhibitors of the RAS/ERK1/2 pathway downstream of RTKs. As the aberrant activity of various RTKs, especially EGFR, plays an important role in the tumorigenesis of human EOC, the present study aimed to examine the expression profile and function of SPRY isoforms in human EOC. Our results demonstrated no significant differences in the mRNA levels of SPRY1 and SPRY4 in different human EOC subtypes. However, the mRNA levels of SPRY2 were significantly decreased in HGSC tissues and human EOC cell lines. In addition, the results of our MIP analysis suggested that deletions in the *SPRY2* gene may be an important factor involved in SPRY2 down-regulation in HGSC. Moreover, we demonstrated that the expression levels of SPRY2 and E-cadherin were positively correlated in HGSC and that the overexpression of SPRY2 attenuated the EGF-induced up-regulation of SNAIL, which, in turn, contributed to the attenuation of the EGF-induced down-regulation of E-cadherin and cell invasion.

Recently, it has been shown that both SPRY1 and SPRY2 mRNA and protein are present in seven different human EOC cell lines. Interestingly, compared to normal OSE cells, four of the seven EOC cell lines exhibited lower SPRY1 and SPRY2 mRNA levels [39]. The results for SPRY2 mRNA are similar to our results, though the results for SPRY1 mRNA are different. In our study, instead of

using normal OSE as an experimental control, we used four SV40 Tag immortalized OSE cell lines as our experimental control. Thus, it is possible that the divergent results are due to the use of two different control cells. The OSE has generally been considered to be the site of cell origin for human EOC [40]. However, recent studies have shown that the fallopian tubes may be where HGSC arises, leading to the current controversy over whether human EOC arises from the OSE or oviductal epithelial cells [41,42]. The results of our HEEBO microarray showed that the SPRY2 mRNA level was lower in HGSC than in the microarray reference. Importantly, although we only included one normal fallopian tube tissue sample, the SPRY2 mRNA level in HGSC was lower than that in normal fallopian tube tissue. Therefore, our results together with previous studies demonstrate that SPRY2 mRNA levels are down-regulated in HGSC.

The human *SPRY2* locus has been mapped to 13q31.1 [26], and previous cytogenetic studies have shown that chromosomal loss of either 13 or 13q is a frequent event in both hereditary and sporadic ovarian carcinomas [43–46]. This frequency can be explained by the existence of known tumor suppressor genes on 13q, including *BRCA2* (13q12-13), *RB* (13q14) and protocadherin 9 (13q21-2). Our MIP copy number analysis results revealed the chromosomal deletion of the human *SPRY2* locus mainly in HGSC and not in other subtypes. In the TCGA database, 16% of serous cystadenocarcinomas showed both gene deletion and reduced mRNA expression of SPRY2. These results supported our finding that SPRY2 mRNA levels are down-regulated in HGSC and that gene deletion is one of the important factors causing this genetic alteration.

It is interesting to note that according to our TCGA gene expression analysis, only 32% of tumors with down-regulated SPRY2 mRNA levels exhibited a gene deletion. These results strongly indicate that additional mechanisms are involved in the down-regulation of SPRY2 mRNA levels. Indeed, the presence of CpG islands in the *SPRY2* 5′ regulatory region suggests the potential involvement of epigenetic mechanisms in the regulation of SPRY2 expression [26]. In both human prostate and liver cancers, down-regulated SPRY2 expression is observed in cancer cell lines and tumor tissues. However, whether SPRY2 promoter hypermethylation contributes to the down-regulation of SPRY2 in human prostate and liver cancers remains controversial [16,23,24,26]. To date, it is unclear whether epigenetic alterations affect the expression levels of SPRY2 in human EOC. Therefore, future studies will be necessary to investigate the status of *SPRY2* promoter methylation and its contribution to the down-regulation of SPRY2 mRNA levels in HGSC.

To the best of our knowledge, the functions of the SPRY protein in human EOC progression remain largely unknown. Only one recent study has shown that SPRY1 overexpression in SKOV3 cells reduces cell proliferation, migration and invasion [47]. We have previously shown that EGF induces SKOV3 cell invasion by down-regulating E-cadherin expression [34,35]. In the present study, we found that the overexpression of SPRY2 in SKOV3 cells did not affect the basal levels of SNAL, SLUG, E-cadherin expression or cell invasiveness. However, the EGF-induced up-regulation of SNAIL, the down-regulation of E-cadherin and increased cell invasion were attenuated by the overexpression of SPRY2. In addition, our results also revealed a positive correlation between SPRY2 and E-cadherin in human EOC tissues. In contrast to our results, SPRY2 has been shown to repress both basal and 1α,25-dihydroxyvitamin D3-induced E-cadherin expression in human colon cancer cells by up-regulating the expression of ZEB1, a transcriptional repressor of E-cadherin [48]. Moreover, the expression levels of SPRY2 and E-cadherin have been shown to be inversely correlated in human colon cancer cell lines and xenografted tumors [48]. These results indicate that the roles of SPRY2 in the regulation of cancer progression are cancer type specific.

Our previous studies have shown that SNAIL and SLUG are two major E-cadherin transcriptional repressors that mediate the EGF-induced down-regulation of E-cadherin [34,35,37,38]. In addition, our previous study demonstrates that the ERK1/2 and PI3k/Akt signaling pathways are involved in EGF-induced SNAIL and SLUG expression, whereas the p38 MAPK signaling pathway only mediates EGF-induced SNAIL, but not SLUG, expression in human ovarian cancer cells [34]. SPRY proteins are well characterized as negative feedback regulators of the RAS/ERK1/2 signaling pathway. However, a recent study demonstrates that SPRY proteins are also able to regulate the activation of p38 MAPK signaling [49]. In mouse embryonic fibroblast cells, targeted disruption of the *SPRY1*, *SPRY2* and *SPRY4* genes enhances the interferon-induced activation of p38 MAPK, though the precise protein target of SPRY proteins among the components of the IFN-activated p38 MAPK pathway remain to be determined [49]. These results suggest that the overexpression of SPRY2 observed in the present study might have affected EGF-induced p38 MAPK activation, which, in turn, specifically attenuated the EGF-induced up-regulation of SNAIL but not SLUG. However, details of the molecular mechanisms regarding this regulation remain to be directly addressed in future studies.

In summary, the present study demonstrates that SPRY2 mRNA levels are down-regulated in HGSC, which may, in part, be caused by chromosomal deletions. The overexpression of SPRY2 reduces EGF-induced cell invasiveness by attenuating the EGF-induced down-regulation of E-cadherin expression. These results indicate that SPRY2 acts as a tumor suppressor in HGSC. Further clarification of the mechanisms underlying SPRY function and regulation will not only advance our understanding of human EOC progression but will also facilitate the development of novel therapeutic strategies.

Disclosure summary

The authors have nothing to disclose.

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